Flora W. Patterson

L.L. Lewis & J.F. Nicholson

Oklahoma Agricultural Experiment Station.

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SOIL INOCULATION.

TUBERCLE-FORMING BACTERIA OF LEGUMES.

L.L. Lewis & J.F. Nicholson

INTRODUCTION.

This work was begun with the idea of working out the cultural characters of the tubercle-forming bacteria of the common legumes, as the subject seemed to be of sufficient importance to justify this, considering the amount of work that is being done on other phases of the subject. A complete knowledge of these organisms and especially a knowledge of their physiology will be of benefit in the practical work of soil inoculation.

After the above work was started, other questions arose in connection with the subject, such as cross inoculation or inoculating one plant with cultures from another, the effect of composition of media and continued cultivation on the activity of the germ, vitality of germ etc. While the above questions have to a certain extent been worked out, others, on account of certain results obtained during the work, have suggested themselves, such as the question of virulence of all cultures, the possibility of increasing the activity of a culture by alternately cultivating and inoculating plants, and the extent to which the organisms multiply

and retain activity in soils not seeded to suitable legumes. It will require considerable time fully to work out and answer some of the questions connected with the subject of soil inoculation and until these are fully understood, it cannot be said that the question of soil inoculation is solved.

Incidentally it is well to state here that in connection with some recent work in this laboratory, cultures of the alfalfa germ that have practically no ability to produce nodules have been isolated while culturally and morphologically the culture could not be distinguished from those that did produce nodules. This culture was obtained in August 1905 and used in a series of pot experiments, but no tubercles were formed

SOIL INOCULATION.

By soil inoculation as here used is meant the addition to the soil of certain bacteria that are necessary to produce nodules on the roots of the leguminous plants. These bacteria are generally very widely distributed but in some localities certain legumes do not form tubercles at all, or they are formed very sparingly and to supply such soil with the suitable bacteria is the object of soil inoculation. Either of two methods may be employed in this work; one is to take soil from an old field that is known to produce nodules on the particular plant desired and apply it to the field to be inoculated, at the rate of from two hundred to five hundred pounds per acre, the other method is to inoculate the seed before planting by using a culture of the desired germ. The latter method is the one that has received so much attention during the past two or three years.

Soil inoculation is not a question of recent development, even in the United States, as some of the experiment stations were conducting experiments along these lines several years ago, notably the Kansas and Mississippi stations. Results reported by the Louisiana experiment station in Bulletin No. 46 are of interest in connection with this subject. Their results seem to indicate that the tubercle-forming bacteria are confined to a few inches of the surface soil. Considering the fact that in cultures, the germ shows a decided preference for oxygen, it would be expected that such would be the case and that the more porous and loamy the soil, or the more it is aerated by cultivation or its mechanical condition is improved by the addition of humus, the deeper the bacteria would be found.

BACTERIA CONCERNED IN THE NITROGEN PROBLEM.

There are two groups of bacteria concerned in the nitrogen problem, one being the bacteria that produce nodules on the roots of the leguminous plants, the other what are known as the nitrifying bacteria of the soil. In their distribution, the second group is found in all soils while the bacteria of the first group are not so widely distributed, as some of the legumes do not form nodules in certain localities.

The nitrifying bacteria:—The group of nitrifying bacteria is divided into at least two sub-groups, one of which is capable of forming nitrites from the ammonia compounds and the second group carries this process still further by forming nitrates from the nitrites, the nitrates being available as plant food,usually in the form of calcium and potassium nitrate. The nitrogen applied to the soil in the form of organic material such as manure, straw or green crops plowed under is not available until the organic compounds have been broken down by the soil bacteria and the nitrogen brought into combination with other substances to form soluble nitrates. This group of nitrifying bacteria is present in all soils and cultivation makes them more active by making the conditions more favorable for their growth.

Tubercle-forming bacteria of the legumes.—The tubercle-forming bacteria are concerned in aiding such plants as the peas, clovers, etc., to secure nitrogen from the air. They are of special importance to agriculture because, of all of the soil elements used as plant food, nitrogen is the most easily exhausted and is the most expensive element to replace in the form of fertilizers. Consequently if these bacteria can be increased in the soil they will benefit directly all such crops as alfalfa, clovers, etc. and indirectly other farm crops.

A few statements are found in the literature relating to the subject of soil bacteriology that the tubercle-forming bacteria will disappear from soils in a few years unless the crop to which they are suited is occasionally grown. This may be true in some cases but certainly does not hold good in all cases. In many localities, these bacteria seem to be normal soil bacteria. This is seen in Oklahoma where the land has only recently been put into cultivation. Plots of land have been examined where sod was broken and planted to cow peas as the first crop and an abundance of nodules was found the first season. Under the head of the report on cow peas is given the results of an experiment to determine the above. As a practical check on the above, unsterilized cow peas have been planted in sterile soil with the result

that only an occasional nodule would be formed, which indicates that the organism is not carried to any great extent by the seed. In connection with the question of the ability of these germs to grow and multiply in soil independent of any legume, experiments have been started to determine to what extent, if any, this is true.

GENERAL PLAN OF EXPERIMENTS.

It is well to state at this time something of the general plan followed in carrying out the series of experiments reported in this bulletin. In order to test the cultures various methods of growing the plants were tried but finally all were abandoned as unsatisfactory except the use of sterile soil in pots. The soil used during the last portion of the work was a mixture of sand, soil and well-rotted manure mixed in equal parts. These pots of soil were sterilized at 350 to 400 degrees Farenheit for three to five hours and the seed was treated with a five per cent solution of carbolic acid for from 30 to 60 minutes. This treatment freed the seed from any bacteria that would interfere with the results and it did not seem to interfere to any great extent with their germination. Sterilized water was used at all times for watering the pots.

No special description is necessary concerning the method of securing the germ from the nodules. The nodules were simply washed clean, usually with a five per cent solution of carbolic acid, rinsed and then mashed in sterile water from which the media were inoculated and plates poured.

Cultures from the alfalfa plant were used in all of the work as outlined below except of course in sections two and three, and in five and nine, where cultures from other plants were used in addition to those from alfalfa. The bacteria from alfalfa were selected, because, in a practical way, this plant seemed of more importance than any other, as alfalfa is probably the most profitable and extensively cultivated legume.

The following topics indicate the divisions of the work and are taken up in the order named.

1. Effect of cultivation and composition of media on the activity of the germ from the alfalfa plant.
2. Experiments with the organism from the cow pea.
3. Experiments with the organism from the soy bean.
4. Experiments with a culture from the Nitro Culture Co.
5. Cross inoculation, or the inoculation of one plant with cultures obtained from another.

6. Normal distribution of the tubercle-forming bacteria of the legumes in the United States.

7. Ability of the tubercle-forming bacteria to increase the nitrogen content of culture media in which they are grown.

8. Sending out cultures.

9. Cultural characters of the tubercle-forming bacteria of the legumes.

10. Conclusions.

EFFECT OF CULTIVATION AND COMPOSITION OF MEDIA ON THE ACTIVITY OF THE GERM FROM THE ALFALFA PLANT.

It is well known that various conditions such as temperature, composition of media and length of time of cultivation, have varying effects on the activity of pathogenic bacteria and it seems reasonable that such conditions would in a measure influence the activity of the bacteria that are concerned in producing nodules on the roots of leguminous plants. In order to determine the effect of the above conditions, experiments were carried out using the germ from the alfalfa plant as this seemed, in a practical way, the most suitable one for the experiments.

The work falling under this head was divided into three series of tests. First, to grow a series of plants in a nitrogen-free nutrient solution and inoculate them with cultures from the alfalfa plant that had been recently isolated; second, to grow plants in pots of sterile soil, inoculating them with cultures that had been grown on a number of different media and that had been transferred a number of times before they were used for inoculating purposes, and finally, to take the cultures giving best results in the second part of the experiment and use them along with cultures recently isolated but grown in media of the same composition.

In the first portion of this experiment, the plants were grown in a nitrogen-free solution and cultures of the germ were added to this. But the liquid cultures added allowed contamination by other bacteria and moulds to such an extent that the plants failed to make a good growth. Good results were obtained only with cultures grown on solid media as the cultures could be removed by scraping the surface of the medium, adding sterile water to the tubes, and using this watery suspension of the germ for inoculating purposes. A number of tests were made at different times in this portion of the experiment but generally with un-

satisfactory results and it was finally abandoned altogether for the method following:

At the time the above test was started cultures were made by inoculating the various media from the same culture as was used to inoculate the plants grown in the nitrogen-free solution.

This gave cultures for the second portion of the experiment having the same source as those used in the first part of the work. These cultures were transferred from the old to fresh tubes as frequently as the growth would permit ,and after they had been transferred a number of times, were used to inoculate the plants in the following experiment. The pots of soil were treated as described in the general outline of technique except that the seed was treated with the five per cent solution of carbolic acid for fifty minutes. The pots were planted to alfalfa on January 11th (1905) and received their first inoculation on January 16th. The pots were inoculated at short intervals during the first ten days, receiving during this time five inoculations. The cultures were grown in test tubes and the amount of culture added at each inoculation was approximately 5 cubic centimeters. The germ was grown on nine different media for the experiment, and four pots were inoculated with the culture from each medium. In order to observe the time necessary for tubercles to form on the roots of the plants and the rate they were forming, the plants were washed out from the pots at intervals of ten, fifteen, twenty-five and thirty days after they received the first inoculation.

It is necessary to include here a brief description of the culture media employed in the experiment.

Agar, potato and Dunham's solution need no description as they were made according to the usual formula used in bacteriological work. The asparagin medium consisted of bouillon plus one per cent of asparagin. Sugar agar had the following composition; water 1000 c. c., magnesium sulphate one-half gram, sugar ten grams, ammonium phosphate eight grams and agar twenty grams. The ammonium phosphate medium consisted of one and one-half per cent of ammonium phosphate added to bouillon. Medium No. 1 consisted of water 1000 c. c., potassium chloride two grams, and one gram each of the following: Sodium chloride, calcium sulphate, magnesium sulphate and calcium phosphate, and cane sugar ten grams. Sugar solution was composed of water 1000 c. c., magnesium sulphate one-half gram, sugar eight grams and ammonium phosphate eight grams. Special bouillon was five per cent of cane sugar added to bouillon.

In addition to the above nine cultures, two others were added, one designated as "alfalfa B," the other as "alfalfa C." These were taken from the same colony isolated directly from the alfalfa plant but were grown in different media. The first was grown continuously in sugar solution while the later was grown on sugar agar.

All of the cultures for this experiment were isolated on the same

kind of medium and then transferred directly to the special media on which they were cultivated continuously until they were used for inoculation. One of the cultures was isolated on September 1st (1904,) the other on October 10th (1904), and both were used for inoculation on January 16th 1905. Four pots of plants were inoculated with each culture. The examinations were made, one pot from each culture, at intervals of ten, fifteen, twenty-five and thirty days after the first inoculation.

The following table shows the results obtained by inoculating alfalfa plants with the above described cultures:

TABLE 1.

Culture Medium Used	No. of Transfer.	Ten Days			Fifteen Days			Twenty-five Days.			Thirty Days.		
		Plants in Pot.	Plants Infected.	No. of Tubercles.	Plants in Pot.	Plants Infected.	No. of Tubercles.	Plants in Pot.	Plants Infected.	No. of Tubercles.	Plants in Pot.	Plants Infected.	No. of Tubercles.
Agar	30	30	0	0	35	3	3	35	5	5	49	7	8
Medium No. 1	30	30	0	0	12	2	2	39	31	82	61	55	241
Potato	27	45	2	2	26	11	15	22	8	14	39	31	65
Alfalfa "C," sug. agr.	16	55	2	4	33	2	2	42	21	36	32	23	51
Alfalfa "B," sug. sol.	20	25	8	9	35	3	3	44	23	48	39	35	127
Asparagin	29	60	0	0	40	2	2	23	3	3	19	3	6
Dunham's solution	29	45	1	1	26	1	1	30	1	2	45	6	6
Special bouillon	29	50	15	25	20	14	24	31	30	71	26	26	183
Ammonium phosphate	23	50	0	0	20	4	7	34	17	26	44	29	54
Sugar agar	29	25	1	2	30	1	1	28	7	12	19	14	31
Check pots		30	0	0	14	0	0	23	0	0	14	2	2

An examination of the above table will show that the number of tubercles formed per plant is very small except with three of the cultures, medium No. 1, sugar solution, and special bouillon. These cultures gave results that are very much better than any of the others, they are all liquid cultures and all contain cane sugar varying from one to five per cent. In regard to their nitrogen, medium No. 1 contained no nitrogen, special bouillon contained nitrogen in the peptone and beef extract used in making the bouillon, while the sugar solution contained nitrogen in the form of ammonia.

In the third part of the experiment, these three cultures giving best results as noted above were continued alongside with fresh cultures grown in media of the same composition and, in addition to the orig-

inal plan, fourteen other media were used, the object being to vary the composition of the media as widely as possible in order to observe the effect on the activity of the germ when judged by the number of tubercles formed. Each culture selected from the previous experiment was continued on the same medium it had been cultivated on and a fresh culture that was isolated just before the previous experiment closed was inoculated into media of the same composition as that used for the three cultures named above. This gave a means of comparing the activity of a germ grown from September 1st to February 24th with that of a culture grown for eight days.

Note. In table II, the media are represented by numbers, with the exception of the first three, which are carried over from the previous experiment. Media designated in the table as 1, 2, and 3 are special bouillon, medium No. 1, and sugar solution and have the same composition as given for the previous experiment. Numbers 4, 5 and 6, bouillon, bouillon plus five per cent glucose, and bouillon plus five per cent lactose respectively. Numbers 7, 8 and 9 are cistern water, each number containing five per cent cane sugar, lactose and glucose respectively; 10, 11, 12 and 13 are bouillon with one-half, one, three and eight per cent of cane sugar respectively; 14, 15 and 16 are 0.2 per cent beef extract with no peptone, 0.01 and 0.5 per cent peptone respectively.

Each culture was used to inoculate four pots of plants, the first inoculation being made on February 24th, 1905. The pots were inoculated with small quantities of the cultures at intervals corresponding to those of the first, with the exception of the pots receiving the old cultures, as they received only one inoculation. (Experiments have been conducted showing that there is little if any advantage in repeated inoculations.)

The following table shows results obtained by using old and fresh cultures that have been grown on media of the same composition, as well as results obtained by growing the germ in a variety of media. In the column under "culture media," A refers to special bouillon, 35th transfer; B to medium No. 1, 34th transfer, and C to sugar solution, 24th transfer.

TABLE II.

Culture Medium.	Ten Days.			Fifteen Days.			Twenty-five Days.			Thirty Days.		
	Plants in Pot.	Plants Infected.	No. of Tubercles.	Plants in Pot	Plants Infected.	No. of Tubercles.	Plants in Pot.	Plants Infected.	No. of Tubercles.	Plants in Pot.	Plants Infected.	No. of Tubercles.
A.	61	36	54	19	11	23	53	53	233	66	66	412
B.	54	45	77	72	68	191	33	32	140	76	76	362
C.	53	36	70	29	23	73	46	45	185	67	65	253
1.	77	40	72	55	52	168	27	27	199	59	59	339
2.	60	40	75	91	83	235	87	83	282	71	71	396
3.	58	50	111	38	37	183	73	69	387	73	73	424
4.	48	33	57	42	42	229	39	39	310	59	59	349
5.	59	41	104	34	27	122	40	40	333	71	71	504
6.	62	50	117	58	55	222	76	73	337	47	47	455
7.	69	59	128	50	50	234	55	55	436	49	49	299
8.	53	47	96	29	29	131	55	54	323	57	57	419
9.	47	30	65	60	54	113	81	79	431	100	100	667
10.	73	40	63	60	60	274	36	36	190	61	57	318
11.	25	8	9	58	46	98	62	56	362	36	34	195
12.	68	36	56	39	36	93	55	54	336	66	66	435
13.	34	27	63	59	57	191	66	64	409	73	73	560
14.	68	36	85	64	53	145	56	56	479	73	73	473
15.	41	23	46	52	43	115	67	65	492	46	46	304
16.	61	39	88	90	72	216	45	44	279	60	59	300
Check	96	1	2	38	0	0	27	2	5	39	2	4

An analysis of results obtained in the last experiment shows that the nodule-forming bacteria of the alfalfa plant may be cultivated for a great length of time on certain media without materially affecting the ability of the germ to produce nodules. For example in table II, media A and 1 are of the same composition. In the former the germ has been grown for several months, being transferred from old to new tubes thirty-five times, while the latter contained a culture taken direct from plates on which it was isolated. The results show their activity to be the same, each giving approximately six nodules per plant. The same is true with reference to the two remaining old cultures B and C and the young cultures 2 and 3, which show approximately the same activity.

In table I the medium giving the best results was bouillon, which of course contained nitrogen, but also contained a high per cent (5 per cent) of cane sugar. Other media rich in nitrogen but containing no sugar gave poor results, as Dunham's solution or asparagin.

From the above results it seems that the presence or absence of nitrogen in the culture media is not the determining factor in main-

taining the activity of the germ. Cultivation in the presence of the amount of nitrogen usually present in bouillon with from two to five per cent of cane sugar or glucose, preferably the former, has given best results in all of the work connected with the experiment.

EXPERIMENTS WITH THE ORGANISM FROM COW PEA.

It has been more difficult to obtain results with cultures from the cow pea than with cultures from any other plant used in connection with this work. In fact we have not obtained nodules on plants in any of the experiments conducted where sterile soil and pure cultures were used. The germ producing nodules on the cow pea is very abundant in the soil of the experiment station farm and seems to be naturally present in the soils of this locality. To test the natural or normal presence of the germ in the soil, a small plot of prairie sod was spaded up and planted to cow peas which had been treated with carbolic acid as described in the general outline of the technique. Plants were examined ten days after they were up and an abundance of small nodules was found on the roots. Examinations made during the time the plants were growing showed that practically every plant was well supplied with nodules.

In making plates from the nodules of the cow pea, it is difficult to obtain cultures having the general characters of those obtained from other legumes. Cover-glass preparations made from the centers of old nodules show an abundance of branch and vacuolated forms, while plates poured from this same material will show relatively few colonies. For a considerable time, a germ was used in these experiments that had all of the general characters of cultures from other legumes, except that it formed a scum in all liquid cultures. When this culture was used in pot experiments,it failed to give any results. After repeated failures with this culture, a viscous colony was found on a plate and used but with no better results. The latter colony produces branched forms as abundantly as alfalfa cultures but so far, no nodules have been formed on plants grown in sterile soil. From the above results it is evident that the tubercle-forming germ from the cow pea was not used in the experiments, or if it was, the manner of cultivating it was such as to destroy its activity.

In April 1905, a culture of the cow pea germ was obtained from the U. S. Department of Agriculture. It was in the form generally supplied from that laboratory and was ready to be used in field work. Portions of this culture or inoculated cotton were used at intervals in

conducting pot experiments but in all cases with negative results. Six tests have been made from material contained in two separate lots of inoculated material and in all cases with negative results.

FIGURE 1.

Photograph of cowpea plant showing nodules on the roots. Taken from a field on the station farm.

SOY BEAN EXPERIMENTS.

The germ producing nodules on the soy bean is not found in the soil of the experiment station farm. There are plots of ground on the farm that have been repeatedly planted to soy beans but in no instance have tubercles been found on the roots of any of the plants examined. In the spring of 1904, some inoculated soil was obtained from the Kansas experiment station and used to inoculate a small plot of ground that was afterwards planted to soy beans. This experiment gave practically negative results as only one tubercle was found on all of the plants that were examined.

A culture of the soy bean germ was obtained from the Department

of Agriculture at Washington during the spring of 1904, cultivated as directed and used in both field and pot experiments with negative results. As only a small plot of ground was to be planted to soy beans, both seed and soil in the row were thoroughly inoculated with the culture, but no nodules were found on any of the plants, and frequent examinations were made from June until September, when the plants were matured.

On August 23rd, 1905, about half an ounce of nodules from the soy bean plant was received from the Kansas experiment station for experimental work. These nodules were washed free from all dirt and thoroughly ground in a small mortar after which a liter of water was added to the pulp. This gave a very white or milky colored fluid which was used to inoculate some pots for pot experiments, also a quantity of seed for field work. The inoculated pots were planted on August 24th and the inoculated seed was planted in the field on August 25th, and was planted in plots alternating with plots planted with seed not inoculated.

One of the inoculated pots was examined on September 13th. The pot contained four plants one of which had twelve nodules varying in size from that of a millet seed to that of a wheat grain. Plants taken from the inoculated plots on the same date showed from one to four nodules per plant while plants from uninoculated plots had none.

This test demonstrates that but a short time is necessary for tubercles to form on the plants if the germ is present in the soil in an active condition, it also indicates a practical and easy way by which inoculations may be made in some cases. The greater portion of the soy beans planted in Oklahoma are planted on wheat stubble. Inoculating material for distribution may be obtained by growing an acre or more of the plants during the early spring in soil that is thoroughly inoculated.. By the time the crop of soy beans is to be planted, inoculating material may be obtained from the inoculated plot. The tubercles may be sent by mail any reasonable distance and arrive in good condition. Three or four ounces of tubercles when thoroughly ground and mixed with two or three liters of water will make an abundance of material for inoculating a considerable quantity of seed as it is only necessary to thoroughly wet the seed. Material for inoculating soy beans and cow peas may be easily and practically obtained in this manner as the nodules formed on these plants are large and a few ounces of them are easily collected. This method would hardly be practicable for clovers, as these plants form very small nodules.

"NITRO CULTURE."

In April, 1905, a package of the culture of the alfalfa germ, put up by the Nitro Culture Co., was secured and used in experiments to determine its value in this work. The cultures are practically in the same form as those put up by the U. S. Department of Agriculture, each package containing a small piece of inoculated cotton and two packages of chemicals which are to be added to a certain quantity of water. Cultures were made from this material according to directions, and pots of sterile soil inoculated. These pots were then planted to alfalfa, using seed that had been treated with 5 per cent carbolic acid for forty minutes. Cultures were also made from the cotton into sterile media in order to determine the character of the culture, that is, whether it was a pure culture or not.

The pot experiments proved that the tubercle-forming germ of the alfalfa plant was present but the results as compared with those from pots inoculated with cultures isolated in the laboratory show that the germ was very scarce in the "nitro culture." A series of six pots was inoculated with the "nitro culture" with the results that 131 plants had 148 tubercles on their roots out of 413 plants that were in the pots. These results when compared with those obtained in Table II give some idea of the relative activity of the cultures.

In the plates poured from the culture, the alfalfa germ could not be found, but showed almost a pure culture of an entirely different organism. This germ when used in pure culture on the alfalfa plant gave negative results. Plates have been poured from every culture obtained from sources other than those isolated from the plant itself and in no case has the predominating organism been the tubercle-forming germ. For these plate cultures, a small particle of the cotton was inoculated into sterile media, in order to secure only such bacteria as were in the cotton.

It is evident from the results, that the "nitro culture" had the alfalfa germ in it and possibly at one time was a pure culture, but foreign bacteria had got into the culture and by the time two or three sub-cultures had been made from the original, the material, as was shown by the plate cultures, was principally something else besides the alfalfa germs.

CROSS INOCULATION.

Since the germs from the tubercles of the various legumes are so much alike in their cultural characters as well as showing a general similarity in preparations made direct from the nodule, the question naturally arises as to whether the bacteria are of one group or species or whether there is a distinct group for each kind of legume. If there is only one group of bacteria, as seems probable, it is entirely possible that those growing on a certain plant, as cow pea, have become so modified by their long continued growth on one plant that they do not readily adapt themselves to other plants, and especially those not closely related. It would not be expected in any case that a culture of bacteria from one plant, as white clover, would produce nodules on alfalfa as readily as a culture from the alfalfa plant, but nodules would nevertheless be formed. This crossing of bacteria from one plant to another is possible in many cases and the following table indicates in a general way the extent to which this may be carried. Moore thinks that it is possible to obtain by cultivation a germ that will form tubercles on any of the legumes.

TABLE SHOWING RESULTS OF CROSSING CULTURES FROM ONE SPECIES OF PLANT TO ANOTHER.

Culture From	Sweet Clover	White Clover	Alsike Clover	Crimson Clover	Mammoth Red Clover	Medium Red Clover	Common Vetch	White Bean	Soy Bean	Alfalfa	Check Pot	Cow Pea
Alfalfa, on	*	-	-	-	-	-	-	*	-	*	-	-
Sweet clover, on	*	-	-	*	-	*	-	-	-	*	-	-
White clover, on	*	*	*	*	*	*	-	-	-	*	-	
(Duplicate test)	*	*	*	*	*	*	*	-	-	*	-	
Common vetch, on	-	-	-	-	-	-	*	-	-	?	-	-
Black locust, on	-	-	-	-	-	-	-	-	-	-	-	
Cow pea, on	-	-	-	-	-	-	-	-	-	-	-	-
White bean, on	*	-	-	-	-	-	-	*	-	*	-	-

*Formed tubercles.
-Did not form tubercles.

NORMAL DISTRIBUTION OF THE TUBERCLE-FORMING BACTERIA OF THE LEGUMES IN THE UNITED STATES.

In order to learn the extent of the distribution of the tubercle-forming bacteria of the common legumes, a letter was sent to each experiment station asking for information in regard to the natural distribution of these bacteria in the soil of their respective localities or states. Some of the legumes are not grown in certain portions of the United States, or are grown so sparingly that no information was received concerning them. This is especially true of the vetches, lupines, and cowpeas in some sections.

Many of the replies were general in their information, stating that tubercles formed on clovers and other feed crops. In such cases the term clovers was taken to include such as the red, white, mammoth red, alsike and sweet clover. Where no information at all was given concerning a given plant, a zero is used in the table while a plus or minus sign indicates that the plant does or does not form tubercles in that locality. In some cases the minus sign may indicate a very slight tendency to form tubercles. Publications from the various experiment stations were used in connection with the correspondence in compiling the table.

There is no doubt but that specific information in many cases would give information not indicated in the table, but in a general way the table indicates the general distribution of these bacteria.

TABLE SHOWING THE GENERAL DISTRIBUTION OF THE TUBERCLE-FORMING BACTERIA OF THE LEGUMES.

STATE.	Alfalfa	Cow Pea	Soy Bean	Vetches	White Clover	Alsike Clover	Sweet Clover	Red Clover	Lupines
Alabama	*	*	*	0	*	0	*	*	0
Alaska	0	0	0	0	0	0	0	0	0
Arizona	*	0	0	0	*	*	*	*	0
Arkansas	*	*	*	0	*	0	*	0	0
California	*	0	0	0	*	*	*	0	0
Colorado	*	0	0	0	0	0	0	0	0
Connecticut	*	*	*	0	*	*	*	*	0
Delaware	0	0	0	0	0	0	0	0	0
Florida	*	*	*	0	*	*	*	*	0
Georgia	*	*	*	0	*	*	*	*	0
Hawaii	*	*	0	*	*	*	*	*	*
Idaho	0	0	0	0	0	0	0	0	0
Illinois	*	*	*	0	*	*	*	*	0
Indiana	*	0	*	*	*	*	*	*	0
Iowa	*	*	-	0	*	*	*	*	0
Kansas	*	*	*	*	*	*	*	*	0
Kentucky	0	0	0	0	0	0	0	0	0
Louisiana	*	*	*	*	*	*	*	*	*
Maine	0	0	0	0	*	*	*	*	0
Maryland	*	*	-	0	*	*	*	*	0
Massachusetts	0	*	*	0	*	*	*	*	0
Michigan	*	0	0	0	*	*	*	*	0
Minnesota	*	*	*	0	*	*	*	*	0
Mississippi	0	0	0	0	0	0	0	0	0
Missouri	*	0	0	0	*	0	*	*	0
Montana	*	0	0	0	0	*	*	*	*
Nebraska	*	*	*	0	0	0	0	0	0
Nevada	0	0	0	0	0	0	0	0	0
New Hampshire	*	0	*	0	*	*	*	*	0
New Jersey	*	*	0	0	0	0	0	0	0
New Mexico	0	0	0	0	0	0	0	0	0
New York	*	0	0	0	*	*	*	*	0
North Carolina	0	0	0	0	0	0	0	0	0
North Dakota	*	0	0	0	*	*	*	*	0
Ohio	*	*	*	*	*	*	*	*	*
Oklahoma	*	*	-	*	*	*	*	*	*
Oregon	*	0	0	0	*	*	*	*	0
Pennsylvania	0	0	0	0	0	0	0	0	0
Porto Rico	*	*	*	*	*	0	*	*	0
Rhode Island	*	*	*	0	*	*	*	*	0
South Carolina	0	*	*	0	0	0	0	0	0
South Dakota	*	0	0	0	*	*	*	*	0
Tennessee	*	*	*	0	*	*	*	*	0
Texas	0	0	0	0	0	0	0	0	0
Utah	0	0	0	0	0	0	0	0	0
Vermont	*	0	0	0	*	*	*	*	0
Virginia	*	*	0	*	*	*	*	*	*
Washington	*	*	0	*	*	*	*	*	0
West Virginia	*	*	*	*	*	*	*	*	0
Wisconsin	*	0	0	0	*	0	0	*	0
Wyoming	0	0	0	0	0	0	0	0	0

ABILITY OF TUBERCLE-FORMING BACTERIA TO INCREASE THE NITROGEN CONTENT OF CULTURE MEDIA IN WHICH THEY ARE GROWN.

A number of media of different composition were inoculated with cultures of the alfalfa germ to determine whether the nitrogen content of the media would be increased after a considerable length of time was allowed for growth. As the work was originally planned, each of the cultures used in the experiment in Table I, was to be transferred to four different media, three flasks of each medium, two of them inoculated and one to be used for a check. The medium for each lot of inoculations was made at one time so there would be no chance for a difference in composition. In addition to the old cultures as indicated, fresh cultures were isolated and inoculated into media of the same composition in order to compare the effects of cultivation on the activity of the germ in this respect. An analysis of some of the flasks seemed to indicate that it would not be necessary to analyze the entire series so that only such flasks were selected as would tend to show the influence that variation of composition of media would have.

The cultures in the experiment had in most cases been carried through certain preliminary work so that it will be necessary to give briefly the history of each culture used in flasks that were analyzed. For convenience the numbers in the following table are divided into groups, each group being inoculated with the same culture.

Groups one and two were inoculated with an old (4 months) agar culture that had been repeatedly transferred. The flasks in group one contained medium No. 1; those in group two contained sugar solution.

Flasks in group three contained medium No. 1 and were inoculated with a culture grown for four months in medium of the same composition.

Flasks in group four contained sugar solution and were inoculated from the same culture as was used for group three.

Flasks in group five contained bouillon and were inoculated from same culture as was used for groups three and four.

Flasks in group six contained sugar solution and were inoculated with cultures direct from plant.

Flasks in group seven contained bouillon and were inoculated with same culture as used in group six.

Flasks in group eight contained medium No. 1 and were inoculated with a culture grown for 31 days in sugar solution.

Flasks in group nine contained cistern water plus 5 per cent glucose and were inoculated with a culture obtained direct from plant.

Flasks in group ten contained sugar solution and were inoculated with a culture grown for 35 days in special bouillon.

In each group two flasks labeled "Sample" were inoculated and the one labeled "Blank" was not inoculated. Each flask contained 100 cc. of culture media.

METHOD OF ANALYSIS AND RESULTS.

(A. G. Ford, Chemist.)

In the determination of the total nitrogen in this series of samples, it was thought advisable to select a method that could be used on all samples alike. On account of the large quantities of nitrogen that were added to certain of the samples in the preparation of the media the use of any of the delicate colorimetric methods was deemed inadvisable. After trials were made with several of the reduction methods on solutions of known composition and strength, the modified Gunning method to include nitrogen of nitrates was selected. The contents of each flask was divided into three parts and each part treated with 40 cc. of salicylic-sulfuric acid mixture. The clear solutions resulting after digestion were combined and made up to 500 cc. Triplicate determinations were made from each of these solutions, using 100cc. for each run. It follows then that the gain in nitrogen represents the difference between the average of three determinations from the blanks and the average of six determinations from the two duplicate cultures.

TABLE SHOWING THE RESULTS OF ANALYSES.

Group.	Number of Flask.	Sample or Blank.	Total N in 100 cc. in mgms.	Gain of N in mgms. per 100 cc. of Sample.	Age of growth in Flask.
	6	Sample	0.28		
1.	7	"	0.00	0.00	53 days
	10	Blank	0.14		
	12	Sample	125.44		
2.	13	"	127.96	2.90	53 days
	15	Blank	123.84		
	40	Sample	Lost		
3.	41	"	5.50	2.70	107 days
	42	Blank	2.80		
	43	Sample	Lost		
4.	44	"	115.50	8.00	107 days
	45	Blank	107.50		
	46	Sample	127.40		
5.	47	"	127.40	0.00	107 days
	48	Blank	127.40		
	112	Sample	31.50		
6.	113	"	30.10	4.20	131 days
	78	Blank	26.60		
	114	Sample	122.20		
7.	115	"	122.20	16.20	131 days
	81	Blank	106.40		
	122	Sample	3.50		
8.	123	"	Lost	3.50	188 days
	84	Blank	0.00		
	133	Sample	3.50		
9.	134	"	1.40	1.05	21 days
	135	Blank	1.40		
	88	Sample	23.80		
10.	89	"	28.70	-0.45	112 days
	78	Blank	26.60		

SENDING OUT CULTURES.

A number of answers from experiment stations, in reply to an inquiry concerning the natural distribution of the tubercle-forming bacteria stated that soil inoculation had been tried to some extent by taking soil from fields that produced good crops of the plant they wished to inoculate. Some referred to the necessity of making inoculations where the legumes formed few or no nodules but did not state whether any work was being done in this line. So far as learned, only one experiment station (Virginia) is sending out cultures. That station supplied them in liquid form for a time, but is now sending out the culture in a dried form on cotton. Drawing conclusions from the experiments with pure cultures from the various legumes, the liquid culture is certainly superior to the dried cultures for distribution.

The germ is very variable in its rate of growtth, especially in media containing little or no nitrogenous material. In the majority of cul-

tures, a good growth is obtained in 48 hours but in some cases a good growth is not obtained for from 3 to 7 days. In all such cases where the culture is used for field inoculation, the bacteria present in the water and from other sources would predominate the culture and cloud the fluid in the same manner as would the germ with which it was intended to inoculate the seed. We have not experimented with any cultures obtained from any source other than from the plant itself where the predominating germ that would develop was the tubercle-forming bacteria of the legumes. In all of the tests with such cultures, sterile media were used and the cotton was transferred as carefully as possible to these media.

By sending out the culture in a liquid form, the germs are in a vigorous growing condition at the time the culture is received. Since the germ is in an active condition of growth, it is much more apt to predominate the sub-culture made by the one intending to use it, than if the germ has to be revived from a dried condition. The liquid culture could be used as soon as received and if the conditions were not satisfactory for seeding, the inoculated seed could be kept, as the bacteria would live as long on the seed in a dried condition as they would on the cotton, provided the seed are kept in the dark.

In connection with the question of the vitality of the germ under the varying conditions of light, drying, etc., experiments were made to determine this. The culture was obtained direct from the alfalfa plant and was then inoculated into bouillon tubes containing pieces of silk thread. After three days' growth, the inoculated thread was removed, placed in tubes and some were then placed in direct sunlight, some in diffused light (laboratory shelf) and the remainder in the dark. Cultures were made at intervals from these threads with the followings results: Threads kept in the dark failed to give a growth after five months, those kept in diffused light failed to give a growth after seventy days while those placed in the window failed to grow after six days. The greater portion of the time that the threads were exposed in the window was cloudy and the germ retained its vitality much longer than it would under ordinary weather conditions. The last part of the experiment was repeated in July by exposing cover-glass preparations to direct sunlight and it was found that none grew after five hours exposure.

The branched and vacuolated forms of the bacteria represent the degenerative stages of the organism and they do not grow and form

colonies. This can be determined only indirectly. Plates poured from the material taken from the interior of large nodules of the cow pea give very few colonies while cover-glass preparations made from the same materials will show an abundance of the branched and vacuolated forms. In fact plates poured from the nodules of any of the legumes worked with, showed that the colonies of the tubercle-forming germ would be relatively few while cover-glass preparations would show the predominating form to be branched and vacuolated. It was also noted that plates poured from cultures containing an abundance of the branched and vacuolated forms gave relatively fewer colonies than those poured from the same culture during early growth or from cultures in media that formed few of the branched or vacuolated forms.

The experiments conducted in testing cultures obtained from various outside sources leads to the conclusion that in some cases the germ sent out is not the tubercle-forming germ at all . In two cases, plates poured from the inoculated cotton gave pure cultures of a germ that was vacuolated, but otherwise did not resemble the tubercle-forming bacteria. Such mistakes could be easily made as there are soil bacteria that produce colonies very similar in general appearance to those of the tubercle-forming bacteria. Since there is the possibility of making the above mistake and also the possibility of obtaining cultures of the desired germ that have no ability to form nodules, it seems that the only way to be certain that the culture is, in the first place the right germ, and second, that it is active, is first to use it in an experiment on the plant from which it was obtained. This can be done without reducing the activity of the germ as is shown by the experiments reported in Table II.

Experiments have been conducted with the dried cultures from alfalfa, red clover, soy bean, and cow pea and in a general way they have failed to give satisfactory results. The cultures from cow pea and soy bean failed to produce any nodules and only a very few nodules were formed from the use of alfalfa and red clover cultures. Such results indicate that there is still something lacking in the practical application of the work and until methods are devised that will give more uniform results, the Oklahoma experiment station will not furnish cultures for distribution in the Territory.

CULTURAL CHARACTERS OF THE TUBERCLE-FORMING BACTERIA OF THE LEGUMES.

Pseudomonas radicicola. (Beyerinck.)

The following description gives data, not only for comparing the cultural characters of the organism from the different legumes, but also a means of comparing the cultural characters of an organism that has been grown in the laboratory for a long time and one that has been recently isolated from the plant. It seems necessary to know definitely the cultural characters of the organisms producing nodules on the legumes in order to be able to form any definite idea as to whether they belong to one group of organisms, modified to some extent on account of long continued growth on one host plant, or whether each legume has its particular organism.

Drawing conclusions from the experiments reported in this bulletin, there seem to be two reasons for believing that there is only one species of these organisms. First, it is possible to take pure culture of such as the alfalfa or white clover organism and produce nodules on other clovers; second, by noting the cultural characters as given in the following description it will be seen that there is very little difference in the cultural characters of the organisms from the different plants, no more in fact than would be expected when bacteria are long subjected to slightly different conditions.

General Statement.—In general, all of the tubercle-forming bacteria that have been worked with occur singly, or rarely in pairs, stain readily with the ordinary aniline dyes, grow readily in ordinary culture media, decolorize by Gram's stain and show a great variation in size and form, depending on the composition of the culture medium and age of the culture.

Two cultures of the germ from alfalfa were used in this portion of the work, one designated as "alfala A," and the other as "alfalfa B." These cultures were isolated at different times and were used in order to see if long continued cultivation would, to any perceptible degree, modify the cultural character of the germ. The culture designated as "Alfalfa A," was isolated in June, 1904, while the culture "Alfalfa B," was isolated in October following. Since the latter culture is more nearly comparable with cultures as they would ordinarily be used in laboratories, the characters of this culture will be given and any differences as compared with the older culture will be noted later.

"Alfalfa B," germ isolated October 10, 1904, investigation begun October 13, 1904.

Relation to Temperature.—The organism grows more rapidly at 38 degrees Centigrade than at 20 degrees or on ice, but will grow at any temperature between these extremes, optimum temperature 28 to 30 degrees C.

Relation to Oxygen.—In stab culture, fermentation tubes or under mica plates, there is only a scanty growth away from free oxygen.

Reaction of Media.—Grows best in slightly alkaline media.

Parietti's Solution.—Growth occurs in bouillon tubes with as much as three drops of the solution added to ten cc. of the bouillon, four drops of the solution prohibits growth. In "Alfalfa A," growth occurred with four drops of the solution to 10 c.c. of bouillon.

Effect of Light and Disiccation.—Inoculated threads were exposed to direct sunlight, to diffused light, and in the dark. In the second test in direct sunlight, growth failed to appear after five hours exposure, in diffused light there was no growth after seventy days exposure and no growth in threads kept in the dark after five months.

Disinfectants.—Used the same method as given in Bulletin No. 62 of the Oklahoma Experiment Station. A one per cent solution of carbolic acid was used. An exposure of one minute was sufficient to prohibit all growth.

Gas Production.—No gas is produced in either glucose or lactose bouillon or in gelatin shake cultures containing the above sugars. Growth occurred only in the open arm of the fermentation tubes and after several days the reaction of the media was slightly acid.

Gelatin Plates.—Plates were kept in ice box at a temperature of 12 degrees C., gelatin neutral. There was no growth noticeable until the sixth day. At this time the colonies were very small and had increased very little at the end of ten days. Deep and surface colonies are much alike.

Agar Plates.—Media neutral, plates kept at 20 degrees C. No growth until the third day when pin point colonies appear. On the sixth day the colonies are finely granular with a smooth border. The deep colonies are lance-shaped and darker than those on top of the media.

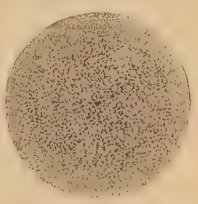

Figure 2. Alfalfa colony from agar plate 72 hours growth at 28 degrees C.

Gelatin Stab Cultures.—Neutral medium, kept at 15 degrees C. No growth appeared until the third day. At the end of ten days the growth was crateriform in shape, gelatin slowly liquified.

Note.—Liquefaction is very slow, in fact evaporation takes place so rapidly that liquefaction is very difficult to determine. In order to know definitely whether liquefaction took place or not, a number of tubes were inoculated and with a number of uninoculated tubes were placed in the incubator. At intervals some of the cultures were shaken in order to distribute the culture through the media and at the end of ten days the tubes were removed and placed in ice water. The inoculated tubes failed to solidify while all of the uninoculated tubes solidified.

Agar Streak.—Neutral medium, kept at 20 degrees C. A slight grayish-white growth along the needle track on the second day. At the end of six days the growth is moist, translucent and with a shiny surface.

Bouillon.—Medium neutral, kept at 20 degrees C. Slight turbidity on the second day. No scum forms at any time but a slight sediment is formed from the sixth to the tenth day. The culture does not clear on standing.

Milk.—There is no visible change produced in litmus milk until about the twentieth day, when it looks slightly watery at the top.

Potato.—No visible growth at the end of ten days.

Nutrient Solution.—This is the medium recommended by the Department of Agriculture at Washington. Medium cloudy on the second day. In "Alfalfa A," growth appeared only after twenty-two days. (This slow growth is unusual, but has been met with occasionally in cultures from the various plants in this as well as other media.

Figure 3. Alfalfa germ from young nodule.

Figure 4. Showing various forms of the alfalfa germ as seen in culture.
1. From 24 hour agar slope.
2. From an 8 day agar slope.
3. From a 48 hour bouillon culture.
4. From a 10 day bouillon culture.

Morphology.—The morphology of the germ varies greatly, depending on the character of the medium and the age of the culture. In some of the cultures, as those containing the sugars, practically all of the organisms are small and solid, even in the 72-hour culture, while in bouillon, agar and especially the old gelatin cultures, vacuolated and involution forms are abundant. Forty-eight-hour bouillon cultures gave approximately sixty per cent vacuolated forms and few branched forms, while in the ten-day culture, practically all are vacuolated and many are branched. The involution forms are abundant in the forty-eight-hour agar culture, but as the culture grows older, these disappear and the small rod-shaped and vacuolated forms appear. The old gelatin cultures gave the greatest variety of vacuolated and involution forms of any media used.

Indol.—Cultures one, two, ten and twenty-two days old gave no test for indol.

Nitrates.—A slight growth present in the nitrate solution but no nitrates formed. (Some cultures will reduce nitrates to nitrites.)

Odor.—No odor produced in any medium.

Pigment.—No pigment produced in any medium.

Enzymes.—A very slow digestion of gelatin and casein.

So far as determined there is no difference between the two cultures from the alfalfa plant used in this test.

The organisms producing nodules on sweet clover, soy bean, white garden bean and white clover were carried through the same plan of cultivation as is reported above for "Alfalfa B," but on account of the similarity in most of the cultural characters, only those will be given that differ from those given above

Organism from Sweet Clover.—This culture was in the laboratory for about five months, but was rejuvenated by cultivation on bouillon, gelatin and agar for three days each.

Bouillon.—A slight scum or ring appears on tubes of six days growth.

Reaction of Medium.—Grows well in either acid or alkaline media but best in alkaline. Growth occurred in agar after potassium hydrate had been added in sufficient quantity to soften the medium.

Nitrites.—Reduces nitrates to nitrites. (Not a constant character.)

Agar Cultures.—Old agar cultures give almost a coccus-like form but agar streaks made from the old cultures gave the characteristic vacuolated form, branched forms being rare.

Organism from soy bean.

Milk.—Slightly changes the color of litmus milk, it is slightly thickened and slowly digested.

Potato.—Growth after fifteen days is moist and brownish in color, turning the potato dark.

Fig. 5. Sweet clover germ direct from young nodule.

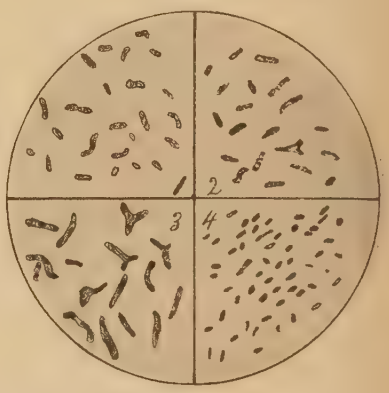

Figure 6. Showing various forms of the germ from sweet clover as seen in cultures.

1. From 18 day gelatin culture.
2. From a 48 hours bouillon culture.
3. From a 48 hour agar slope.
4. From a 20 day agar culture.

Reaction of Medium.—Grows in either acid or alkaline media but slightly better in acid, differing in this respect from the germ from alfalfa and to a less degree from the organsm from sweet clover. Produces no acid in growth.

Nitrites.—Reduces nitrates to nitrites.

Organism from White Bean.—

Milk.—Slowly digest the casein.

Potato.—Very slight white growth after ten days but does not change the color of the potato.

Reaction of Medium.—Appears to grow equally well in strong acid or alkaline media.

Nitrites.—Does not reduce nitrates to nitrites.

Organism from White Clover.—

Potato—A scanty dirty-white growth after six days. Growth shows many large vacuolated forms.

Enzymes.—Gelatin slowly liquified but no apparent effect on milk.

Nitrites.—Production of nitrites is not uniform, some cultures failing to give the test.

Figure 7. Showing germ from white clover in cultures and from nodule.

1. From a 25 day agar culture. (Sugar agar.)
2. Germ direct from nodule.
3. From 5 day agar slope.
4. From 30 day culture in bouillon plus 3 per cent glucose.

Figure 8. Showing germ from red clover and cow pea.

1. Germ direct from nodule.
2. From a 10 day agar culture.
3. From center of old tubercle of cow pea.
4. From very young tubercle on cow pea.

The important differences in the cultural characters of the above germs is their variability towards acid or alkaline media, their differences when grown on potato and their variation in the reduction of nitrates to nitrites. The morphology varies so greatly under different conditions that it is useless to attempt a description. As an illustration of this variation, the following from the notes on white clover is given: In the nitrate solution the germ is very small, averaging from one-third to one-half micron in diameter, a few clubbed forms, but branched forms rare. When grown in Dunham's solution the germ is very large, some from one to one-half microns in diameter with many vacuolated forms. This variation in size and shape is noted in all of the germs studied.

CONCLUSION.

In the long series of pot experiments that have been carried out in connection with this work, it has been a noticeable fact that tubercles formed more readily and more abundantly in fertile soil than in soil poor in plant food. In sand the tubercles would form on the plants when inoculated with either pure cultures or soil infusion, while in pots containing sand, normal soil, and well rotted manure mixed in equal parts and treated in other respects as the pots of sand, tubercles would form more quickly and in greater numbers than in either sand or normal soil. In all of the tests during the later part of the work the above mixture of soil, sand and manure was used, which in itself would insure a vigorous plant growth and at the same time give a more abundant tubercle formation Repeated experiments have shown that with the same amount of inoculation the plants grown in fertile soil will form more nodules than those grown in sand.

It should not be assumed that these nodule-forming organisms can replace the necessary plant food, proper cultivation of soil and other things that are regarded as necessary for growing crops. If the soil is poor, the legumes will make a poor growth even if the proper germs are present, and no amount of inoculation will produce crops equal to those grown on fertile soils. If other factors necessary to plant growth are favorable, and tubercle-forming bacteria are absent or even present in small numbers, then soil inoculation may prove beneficial. The mechanical condition of the soil, together with the amount of humus present, are great factors in growing alfalfa, and in many cases the poor upland soil is deficient in humus and does not lend itself to a good state of preparation, hence the failures where attempts are made to grow alfalfa on soil of this character.

Information received from several of the experiment stations shows that the practice with them is to furnish plenty of humus in the form of manure and when this is done, a good growth of alfalfa is generally secured, even on poor lands, as in nearly all localities there are sufficient bacteria present to inoculate the crop, if the ground is in such condition as is necessary to insure a good plant growth. There should be reason and moderation in all things and just now a great many are wanting to grow alfalfa on all kinds of soils by applying bacteria, when little or no preparation has been given to the soil in order to have a

good seed bed. Such ideas and practice is clearly an overestimation of the possible good of soil inoculation.

There seems to be prevalent to a great extent the idea that if the tubercle-forming bacteria are lacking in a soil that it is useless to undertake to grow the particular crop for which they are wanting. This is certainly far from the observed facts. Heavy yields of soy beans are obtained from crops grown on the experiment station farm and there are no nodules formed on the soy bean in this soil. However, the crop is grown on soil that has an abundance of plant food and is well cultivated. Other things being favorable, a rich soil in good mechanical condition will produce a good growth of alfalfa whether the nodule-forming bacteria are present or not. The lack of tubercle-forming bacteria is more noticeable on poor soils than on fertile soil. In the pot experiments the growth of plants in poor soil receiving inoculation was better than in pots not inoculated, but in those pots receiving a mixture of sand, soil and manure, the growth was as vigorous in the check pots as in those inoculated and no difference could be noticed at the end of fifty-five days, which was the greatest length of time any of them were allowed to grow. The inoculation is only one step in successfully growing legumes and no matter how carefully carried out, will fail, if other things, just as essential, have been omitted.

The experiments carried out in connection with the tubercle-forming bacteria of the legumes justify the following conclusions:

1. Soil inoculation with cultures of the tubercle-forming bacteria is practicable only when other conditions are favorable for plant growth.

2. It is possible to grow these bacteria for at least thirty to thirty-five generations on special media without materially lessening their activity.

3. Some cultures examined in the laboratory purporting to be cultures from the alfalfa plant, do not contain the germ from the alfalfa plant at all, but are cultures of a soil organism resembling the germ from alfalfa in some particulars.

4. The tubercle-forming bacteria of the legumes are easily destroyed by light, consequently inoculated seed should be kept in the dark until used.

5. Since some of the ordinary soil bacteria form colonies very similar to those of the tubercle-forming bacteria and also resemble them to a certain extent in being vacuolated, every culture, from which subcultures are to be made for distribution, should be tested in pot experiments.

L. L. LEWIS,
Veterinarian.

J. F. NICHOLSON,
Assistant in Bacteriology.

(W. R. Wright, who suceeded J. F. Nicholson as assistant in bacteriology in August, 1905, assisted in checking the results reported in this bulletin.)